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<p>During metastasis from the primary tumor, cell motility is believed to have an important role in the dissemination of tumor cells. Isolating the motile population of cells from a tumor allows for a unique way of studying the mechanisms of cell motility involved in intravasation. We have developed a matrigel filled catheter that when placed into a tumor mimics the extracellular and vessel environments found in tumors. With this microneedle catheter we are able to selectively collect up to 1000 cells in needles that contain 25 nM EGF or 50,000 units/ml of CSF-1. By amplifying the RNA isolated from these cells we will be able to use microarrays to do gene discovery to find gene products that are associated with cell metastasis and motility. These gene products then can be used as targets for clinical and pharmaceutical research to further understand and prevent metastasis.</p>			
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## **Introduction**

Metastasis represents the major cause of mortality in breast cancer patients. Components of the metastatic process can include growth at the primary site, attraction of new blood vessels to the primary tumor, dissemination of tumor cells in the surrounding connective tissue, intravasation and distribution of tumor cells in the bloodstream or lymphatics, spread to axillary lymph nodes, extravasation across blood vessel walls and into the tissue parenchyma at a secondary site, and growth at the secondary site. These components encompass and rely upon a large number of cellular functions. Cell motility is believed to be important for dissemination of cells from the primary tumor, intravasation and extravasation. Proof that the motility of tumor cells is a key component in metastasis would be a major advance.

We have developed a collection process that allows for the selective isolation of the motile population of tumor cells from a living breast tumor. By using an imaging technique we developed, we have been able to identify the motile cells within a tumor and shown that intravasation plays a major role in the dissemination of cells through metastasis (1, 2). By developing a catheter based collection process, we are able to collect up to 1000 cells from these tumors by creating an environment that mimics the vessels and matrix within a tumor. By filling a microneedle with matrigel and a chemoattractant such as EGF or CSF-1, we are able to attract the cells to crawl passively into the catheter. These 1000 cells can then be used for RNA amplification and microarray analysis that will allow for the gene discovery of genes and gene products important to cell motility and metastasis.

## **Body**

The original reported data from the first year was accomplished in an orthotopically injected tumor using MTLn3 and MTC adenocarcinoma cells. These cell lines represent a well characterized cell pair which were derived from the same original tumor and which retain their relative metastatic phenotypes after prolonged culture (3, 4). Examination of the behavior of cells in the MTC (non-metastatic) and MTLn3 (metastatic) derived tumors demonstrates significant differences between the two. As we have shown previously, there are significant differences between the morphological and intravasation properties of the two tumor types. By using cells labeled with green fluorescent protein (GFP), we were able to image cells interacting with the host environment and evaluate differences between the two tumors (2). While both cells show the same cell motility and protrusion within the tumor, the MTLn3 generated tumors show more cell orientation toward vessels, more intravasation of living cells (cells in the blood), and more host cell involvement, while the MTC generated tumors showed more cell fragmentation upon entering vessels (2). By moving to a multiphoton microscope, we have been able to reconfirm these findings and improve image quality. Also we are able to image at a greater depth; while also imaging the extra-cellular matrix due to

autofluorescence in the UV. Multiphoton microscopy works by using longer wavelengths of light which upon intersection at the plane of focus allows excitation of a chromophore at that point only, allowing for deeper penetration and less bleaching and cell toxicity. We have observed that cells directly along a vessel will show a high degree of locomotion, while those away from the vessels tend to show little or no movement.

In the preliminary cell collection studies, we were able to show a four-fold increase of MTLn3 cells in microneedles containing 25nM EGF and matrigel over needles with matrigel alone and 15-fold increase in the amount MTLn3 cells over MTC cells in microneedles containing 25nM EGF and matrigel (5). By decreasing the concentration of matrigel in the needles from 1:2 to 1:10 dilution in L15 medium containing .39% BSA, the number of cells collected increased 50-fold. In a dose response curve, the collection was shown to be selective for EGF concentration as 21-fold more cells were collected in needles containing 25 nM EGF than those with no EGF, and at 50nM EGF the number dropped to around 11-fold over no EGF needles (Figure 1).

To make the model more clinical, the orthotopically injected tumor cells in the rat were switched to transgenic mice containing the genes for MMTV-GFP x MMTV-PyV middle-T antigen were used. The oncogene for middle-T has been shown to be clinically relevant in comparison to human breast cancer disease (6). By histology, it was shown that tumors containing GFP are identical morphologically to tumors with the middle-T antigen alone (Figure 2). By using the same intravital imaging technique using the multiphoton microscope, cells at varying stages of metastasis can be identified by GFP fluorescence and can be confirmed by similar areas of histological sections (Figure 3).

In the cell collection from these spontaneous tumors, 1000 cells were collected into microneedles containing 25nM EGF and matrigel and in needles containing CSF-1 at 50,000 units/ml (Figure 4). Matrigel-alone microneedles contained approximately 200 cells. CSF-1 was chosen because it has been shown that CSF-1 interaction with macrophages promotes the progression of tumor malignancy (7) and promotes metastasis (8). To test for cell specificity, mice containing MMTV-PyV middle-T x lys-GFP<sup>Ki</sup>. Lys-GFP<sup>Ki</sup> mice produce GFP labeled granulocytes. By counting cells stained with DAPI and GFP fluorescent cells, granulocytes collected can be differentiated from other cell types. For 25nM EGF containing microneedles, of the 1000 cells collected 10 % were GFP fluorescent. In the 50,000 units/ml CSF-1 containing needles, approximately 30% the cells were fluorescent. The ratio for matrigel alone was similar to that of the EGF containing needle (Figure 5). The increase in granulocytes in the CSF-1 containing needle can be attributed to the role of CSF-1 in the promotion of metastasis (8).

The RNA isolated from these 1000 cells can be amplified using SMART cDNA technology (Clontech) for microarray gene discovery. By comparing gene differences between the collected cells and cells from the whole tumor, gene products specific to the motile population of cells can be identified. We have tested the procedure using 1000 MTLn3 cells in comparison with 1000 MTC cells and shown that the amplified RNA is equivalent to the non-amplified sample. This has been further confirmed by real time (RT) PCR (data not shown).

equivalent to the non-amplified sample. This has been further confirmed by real time (RT) PCR (data not shown).

As of now, cells are being identified by DAPI staining and GFP fluorescence. Also, cells are identified using markers specific for macrophages and endothelial cells. Task 3 has yet to be undertaken, but cell specificity and identity can be completed as described above.

### **Key Research Accomplishments**

- Further development of catheter cell collection technique to increase cell number.
- Cell specificity has been determined by chemoattractant dose response curves and GFP fluorescence.
- Creation of spontaneous tumor bearing mouse models as a better correlation to human disease.
- Cell specificity and metastatic correlation for cells collected using GFP-tagged granulocytes and CSF-1 as a chemoattractant.
- Early results of molecular determination of collected cells.

### **Reportable Outcomes**

#### Manuscripts:

Wyckoff, J., Segall, J., and Condeelis, J. (2000) The collection of the motile population of cells from a living tumor. *Cancer Research*, 60:5401-5404.

#### Abstracts and Presentations:

Wyckoff, J., Frohlich, V., Jones, J., Segall, J., and Condeelis, J. (2000) Imaging of primary tumors in whole animals using laser-based tomography. Abstract and presentation, San Antonio Breast Cancer Symposium.

Wyckoff, J., Bailly, M., Jones, J., Condeelis, J., and Segall, J. (2000) The rate-limiting step in metastasis: In vivo analysis of intravasation at the primary tumor. Abstract and presentation, San Antonio Breast Cancer Symposium.

#### Career Development:

Grants and other funding received:

PAR-99-100, R21/R33 NIH grant. Innovative technologies for the molecular analysis of cancer: phased innovation.

## Conclusions

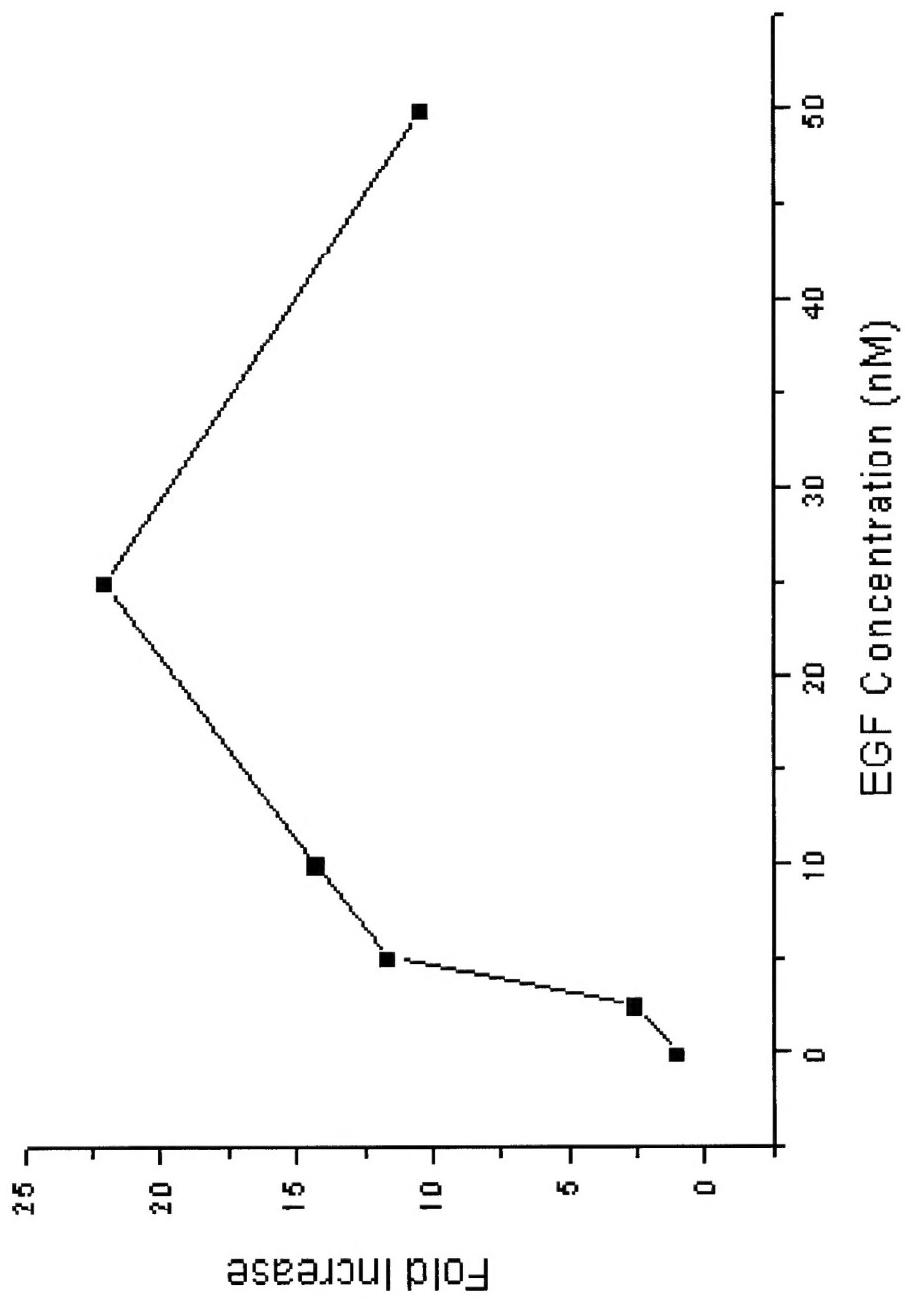
By improving the technique of catheter cell collection and changing from an orthotopic injection model to a transgenic model has greatly increased the cell number of collected motile cells and helped with further identity of cell specificity. By using antibodies to specific cell markers, we will be able to further achieve the cell specificity needed to perform gene discovery on these cells. New advances in cDNA amplification and our own results show that the number of cells that we are collecting will be enough to perform microarray gene discovery. This will lead to the ability to find and explore genes important to cell invasion, motility and metastasis that in the future may lead to more targets in the fight against cancer.

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**Figures and Appendices**

(see attached)

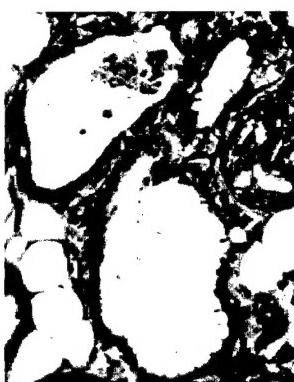


**Figure 1: A dose response curve of EGF for the collection of carcinoma cells from a primary tumor of the breast shows that the collection of the motile population of cells is EGF concentration dependent.** By using microneedles filled with matrigel and varying concentrations of EGF, a 20-fold increase of motile cells can be seen at 25nM over matrigel alone. At 50nM EGF, the number of cells decreases, suggesting the inhibition of motility by overloading the REGF receptors.

**MMTV-PyMT**



**MMTV-GFP x  
MMTV PyMT**



**Adenoma**

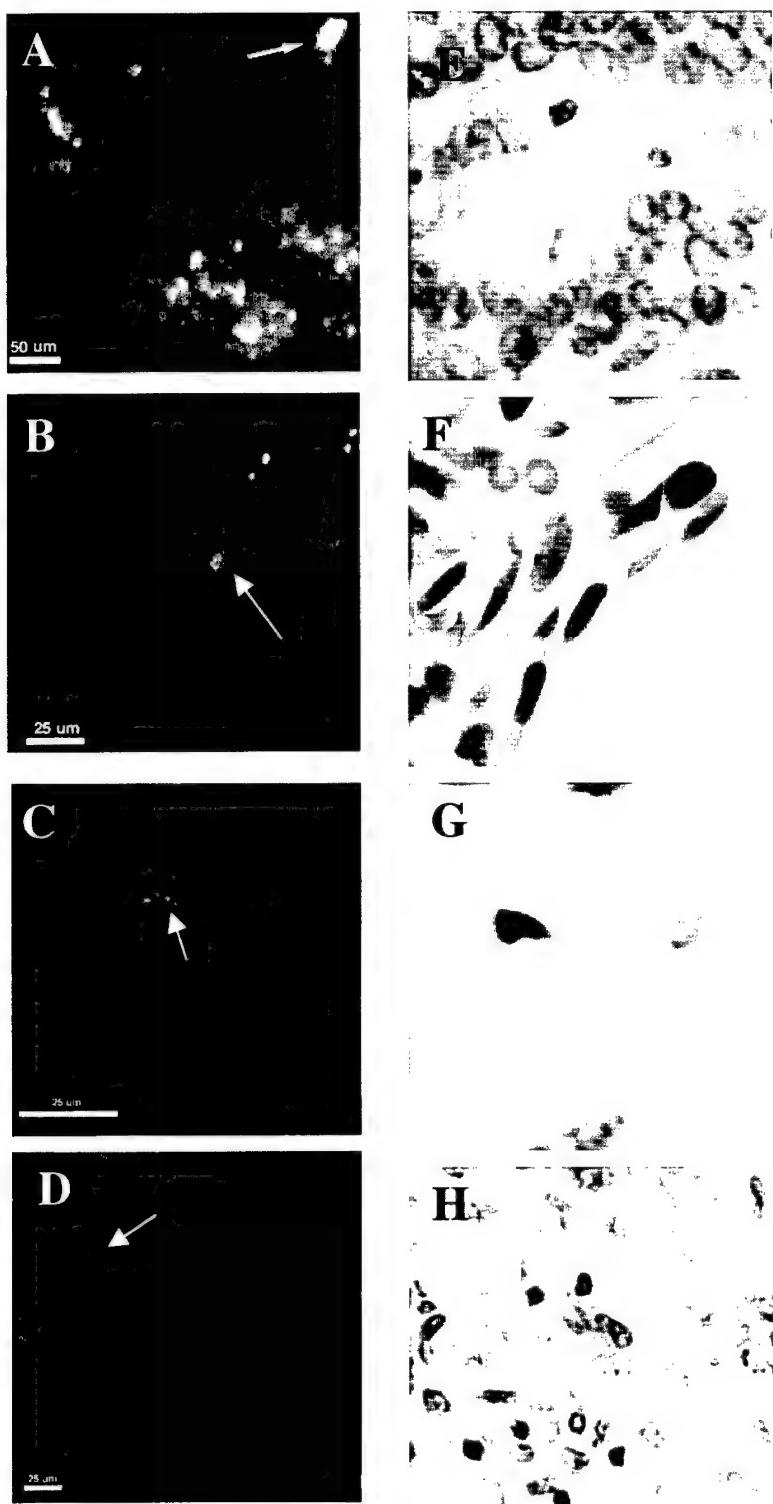


**Early Carcinoma**

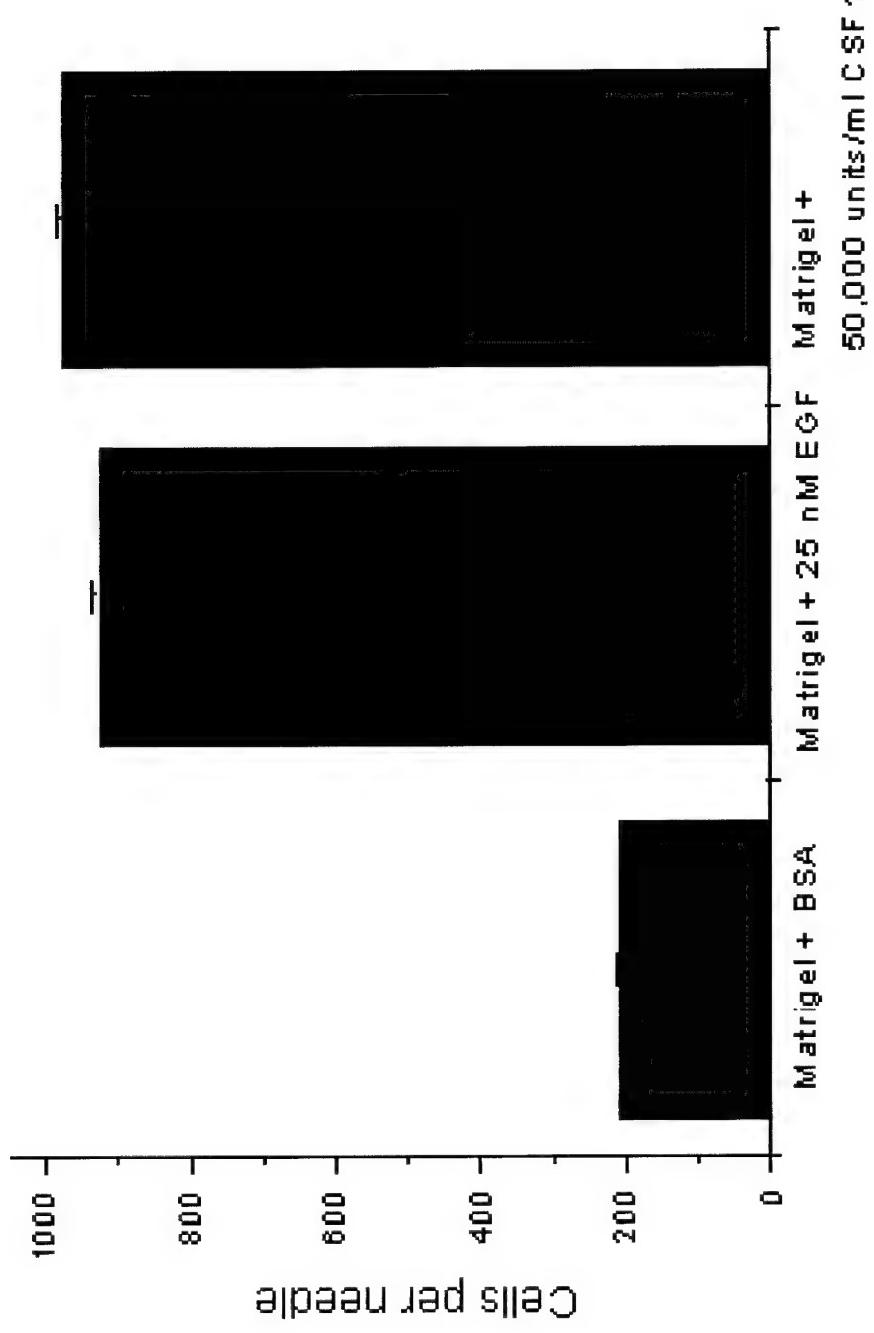


**Late Carcinoma**

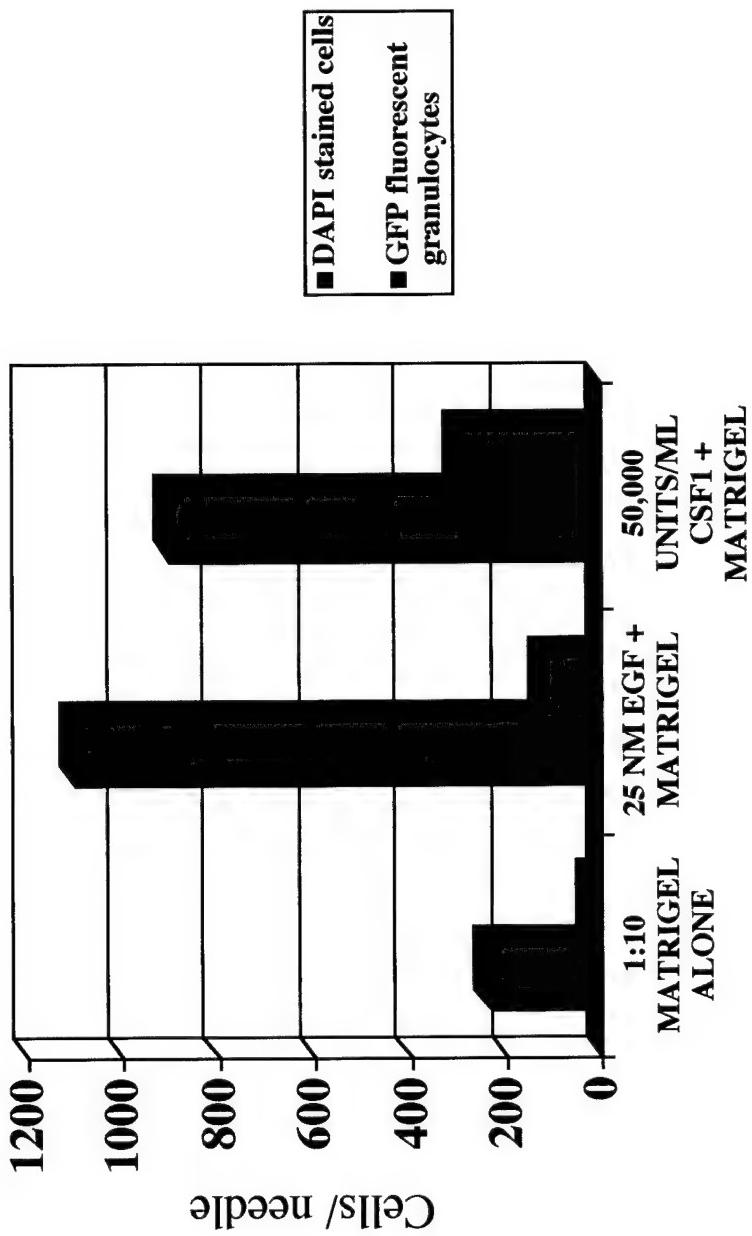
**Figure 2: Comparative histology between breast tumors generated in transgenic mice containing only the MMTV-PyMT gene and those containing MMTV-GFP x MMTV-PyMT.** Tumors from mice that have the GFP gene along with the middle T antigen gene go through the same developmental stages as those that do not have the GFP gene.



**Figure 3: Multiphoton microscopy can be used to image GFP fluorescent living tumor cells in transgenic mice with the MMTV-GFP x MMTV PyMT. A-D)**Tumor cells containing GFP (green) imaged using a multiphoton microscope. Arrow points to a single cell. Cells can be seen interacting with extracellular matrix (blue), and lining vessels and fat cells. **E-H)** Histological section of similar areas within GFP expressing tumors can be used for morphological comparison to living tumor cell images.



**Figure 4:** There is a five fold increase in the number of cells collected in needles containing matrigel with either 25nM EGF or 50,000 units/ml of CSF1 than in needles with matrigel alone. By using microneedles filled with matrigel and chemoattractants, up to 1000 cells per needle can be collected that represent the motile population of cells in a tumor. N = 4 (ie 4 experiments in 4 different animals with breast tumors)



**Figure 5: Collection of invasive cells from primary breast tumors in MMTV-PyMT x lys-GFPki mice.** There was a 6-fold and a 4-fold increase of cells collected in microneedles containing EGF and CSF-1, respectively, than in matrigel alone. Only about 1:10 of the cells in the EGF containing needle were GFP positive granulocytes, while approximately 1:3 were GFP positive in the CSF-1 containing needle. This suggests that cell type selectivity has occurred with EGF attracting mainly carcinoma cells and CSF1 attracting both granulocytes and carcinoma cells.

## The Collection of the Motile Population of Cells from a Living Tumor

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### Abstract

In this study, we report that needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor in a live rat as a pure population suitable for further analysis. The most efficient cell collection requires the presence of chemotactic cytokines, such as epidermal growth factor and serum components, and occurs with 15-fold higher efficiency in metastatic tumors compared with nonmetastatic tumors. Although tumor cells of the non-metastatic tumors show a motility response to serum, they were not collected with high efficiency into needles *in vivo* in response to serum, indicating that additional factors besides motility are required to explain differences in cell collection efficiencies between metastatic and nonmetastatic tumors. The results reported here indicate that needles filled with growth factors and matrigel, when inserted into the primary tumor, can faithfully mimic the environment that supports invasion and intravasation *in vivo*. Furthermore, the results indicate that the same cell behaviors that contribute to chemotaxis *in vitro* also contribute to invasion *in vivo*.

### Introduction

Metastasis involves the escape of cells from the primary tumor either via lymphatics or blood vessels, transport to and arrest in a target organ, and growth of metastasis in the target organ (1). Each of these steps is a multicomponent process, with potentially different tumor cell properties and molecules playing critical roles at different steps (2). Recently, emphasis has been on the development of molecular arrays to identify new genes and proteins that contribute to specific steps in metastasis. Such approaches are crucial in the analysis of cancer as a genetic disease and in the identification of key genes that might be used in diagnosis and therapy. However, array-based approaches treat the tumor as a black box. Ideally, high-resolution methods for the analysis of metastasis at the cellular level, such as imaging of cells within tumors, when combined with array-based approaches, could be used to accurately evaluate the roles of specific gene products in the individual steps of metastasis at the cellular level. The use of Laser Capture Microdissection as a front end for array-based gene discovery is such a fusion approach (3). However, some of the cell behaviors that are believed to be essential for metastasis, such as adhesion and motility (4, 5), cannot be used as criteria in the selection of cells for analysis from fixed material because the behavior and history of individual cells cannot be inferred from fixed material. Methods for the collection of cells from living tumors in which key cell behaviors can be observed and used as the criteria for cell collection need to be developed. One such cell behavior is the chemotaxis of tumor cells. Metastatic tumor cells are believed to chemotax to cytokines that are normally found in association with blood vessels (6–8). We developed a cell graft breast tumor metastasis model in rats that is syngeneic and orthotopic that

permits the imaging and tracking of cell behavior in live tumors (8, 9). Using this model, we have observed, in metastatic primary tumors, the highly persistent linear locomotion of a subpopulation of tumor cells toward blood vessels *in vivo* using intravital imaging. This locomotion resembles the chemotaxis of cells observed in culture (9, 10) and is correlated with metastatic potential (10, 11). Tumor cell chemotaxis is also correlated with the accumulation of metastatic tumor cells around, and their polarization toward, blood vessels in the primary tumor. Furthermore, chemotaxis is correlated with the efficient intravasation into, and survival of tumor cells in, the systemic circulation (8). Because these properties are not observed in nonmetastatic tumors prepared from cells in the same way (7, 8), polarization and chemotaxis toward blood vessels are believed to be important in intravasation and metastasis (8). In this study, we report that chemotaxis can be used to advantage to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor *in vivo* as a pure population suitable for further analysis.

### Materials and Methods

**Serum Upshift of Cells *In Vitro*.** MTLn3-GFP and MTC-GFP cells were plated in 35-mm dishes at a density of 50,000 cells per dish 18 h before the experiment. On the day of the experiment, cells were starved for 3 h in 2 ml of MEM containing HEPES and 0.6% BSA, which is the isotonic equivalent of 10% FBS<sup>2</sup>. The upshift was performed as described before (12), with the exception that the cells were stimulated with 10% FBS. Briefly, the dishes were covered with a thin layer of heavy mineral oil (Sigma #400-5) and placed in an enclosed microscope preheated to 37°C. Using a CCD camera, single-frame images were collected using NIH Image every minute. After 4 min, 2 ml of MEM with HEPES and 20% FBS were added to the dish, and image frames were collected for an additional 16 min.

**In Vitro Cell Collection.** MTLn3 cells were plated in a 35-mm dish 18 h before the experiment to be 60–80% confluent at the time of the experiment. On the day of the experiment, cells were starved using MEM-BSA, the isotonic equivalent of 5% FBS, for 2 h. During this time, 26-gauge syringe needles were prepared by filling them with 10 μl of Matrigel mixed 1:1 with MEM-BSA or MEM-BSA containing EGF for a final concentration of 0.5 nm, 2.5 nm, 5 nm, 25 nm, 50 nm, or 250 nm EGF. After starvation, the needles were attached to the side of the plate using paraffin to hold them in place with the bevel of the needle facing the bottom of the plate so that the matrigel was in contact with the surface of the plate. Dishes were placed into a 37°C/5.0% CO<sub>2</sub> incubator for up to 6 h. After this time, the contents of each needle was extruded into a new 35-mm dish containing MEM with 5% FBS (growth medium). Cells that had entered the needle were allowed to grow into clones for 6 days to determine cell count and viability. Positive clones, checked by GFP fluorescence and cell morphology, were then counted.

To image the cells moving toward the needle, a dish was plated for 40–50% confluence before the experiment. Cells were starved, and a needle was prepared as above containing matrigel mixed 1:1 with MEM-BSA containing 25 nm EGF. Images as single frames were taken using the heated microscope and NIH Image every 30 min, as described above. The dish was kept in a 37°C/5% CO<sub>2</sub> incubator between images.

**In Vivo Cell Collection.** MTLn3-GFP and MTC-GFP cells were injected into female Fischer 344 rats, as described before (8, 9), and tumors were

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<sup>2</sup> The abbreviations used are: FBS, fetal bovine serum; MEM-BSA, MEM with 0.35% BSA; EGF, epidermal growth factor; i.d., inside diameter; GFP, green fluorescent protein.

allowed to grow for 2.5 weeks. On the day of the experiment, 33-gauge needles were prepared as above by filling them with matrigel and MEM-BSA, MEM-BSA with a final EGF concentration of 25 nM, or MEM-BSA with a final FBS concentration of 10%. All needles included 0.01 mM EDTA (pH 7.4) to sequester heavy metals that might be released by the needle. A rat was anesthetized using 5% isoflurane and laid on its back. The isoflurane was reduced to 2%, and a small patch of skin over the tumor was removed. Three 25-gauge needles (guide needles) with blocking wires were inserted to a depth of 2 mm. The blocking wire was removed, and one of the matrigel-filled needles was inserted into each guide needle (as shown in Fig. 3). The needle was then left in the tumor for 6 h. The isoflurane concentration was slowly lowered to 0.5% during the course of the experiment to keep the rat's breathing even and unlabored. After 6 h, the needles were withdrawn, extruded into 35-mm dishes containing growth medium, and all cells were counted immediately. The percentage of cells with GFP fluorescence was determined.

As a control for the effects of matrigel, a 33-gauge needle was filled as above with MEM-BSA and agarose, for a final concentration of 1%, and the *in vivo* experiment was performed as above.

## Results

**In Vitro Cell Collection.** As has been shown previously, MTLn3 cells are chemotactic to EGF with an optimum concentration at 5 nM EGF (12). Also, it has been shown that MTLn3 cells, when placed in a gradient generated using a pipette filled with 50  $\mu$ M EGF, will orient toward and locomote in the direction of the pipette exhibiting true amoeboid chemotaxis (10). MTLn3 cells are metastatic when re-injected into the mammary fat pad of a Fischer 344 rat. We prepared an artificial microenvironment using microneedles filled with matrigel and either EGF or serum as the chemoattractant to simulate invasion and intravasation into a container that could be withdrawn to collect the chemotactic/invasive subpopulation of cells.

To establish the concentration necessary to attract MTLn3 cells into the needle, needles were filled with a range of EGF concentrations from 0.5–250 nM and inserted into a cell culture. At times up to 6 h of collection, the needles were withdrawn from the culture and the contents were extruded into a new dish with growth medium, and the cells were allowed to grow for 6–7 days to determine cell counts and test viability. The number of cells entering the needle was determined by the number of GFP fluorescent clones that grew during this time. At the peak concentration of 25 nM EGF, an 8-fold increase in the number of cells entering the needle was seen, when compared with buffer alone (Fig. 1). The number of cells collected decreased at 50 nM EGF, and by 250 nM EGF the number of cells collected returned to near background.

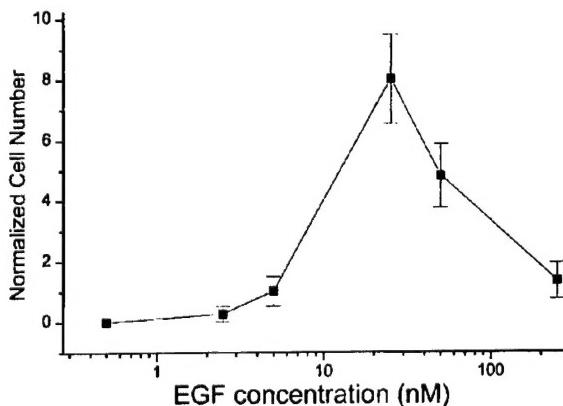


Fig. 1. Tumor cells are collected into matrigel-containing needles in response to EGF. Cells in culture were collected in needles containing matrigel and differing concentrations of EGF. The maximal number of cells was collected into the needle containing 25 nM EGF. Cell numbers were normalized to MTLn3 cells collected with matrigel in buffer. Bars, the SE of three experiments.

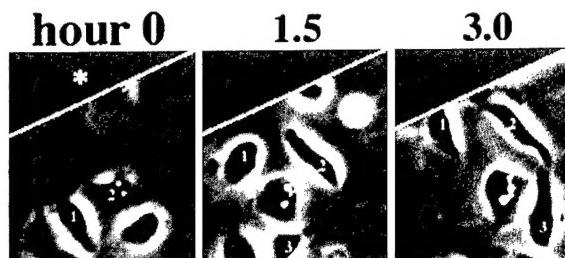


Fig. 2. Tumor cells chemotax toward needles containing EGF. Cells are seen orienting and moving toward a needle containing matrigel and 25 nM EGF. By 1.5 h after the needle was placed in the culture dish, cells 1 and 2 have already oriented themselves toward and have moved in the direction of the needle, whereas cell 3 has entered the field of view. By 3 h, cells 1 and 2 have reached the matrigel edge. The edge of the matrigel (\*) is delineated by the white line and shown in gray. Only motile cells within the field are numbered. The average velocity of the cells is  $0.32 \pm 0.03 \mu\text{m}/\text{min}$ .

The differences in EGF concentration optimum for cell response between the upshift (5 nM; Ref. 12), the pipette experiment (50  $\mu$ M; Ref. 10), and the collection experiment reported here (25 nM) can be explained by the differences in diffusion of EGF in the different experimental designs. In the upshift, there is no gradient involved and the cells see an equal and constant concentration of EGF. For the pipette experiment the gradient is created by a pipette with an i.d. of  $<1 \mu\text{m}$ , and the concentration outside of the pipette is only a fraction of the concentration in the pipette. For the *in vitro* cell collection experiments reported here the i.d. of a 26-gauge needle is 250  $\mu\text{m}$ ; hence, a larger percentage of EGF is delivered per unit time so that a much lower EGF concentration is necessary than in the pipette experiment (10).

By using a needle loaded with matrigel and 25 nM EGF in MEM-BSA, we were able to capture images of the cells moving toward the pipette, using time-lapse video-microscopy. In Fig. 2, the matrigel surface at the edge of the needle is delineated by the *white line* and colored *gray*. At time zero, cells 1 and 2 are seen as nonpolarized cells with no discernable leading edge. After 1.5 h, cells 1 and 2 have oriented themselves toward and moved in the direction of the needle-induced EGF gradient, extending a leading lamellipod toward the needle. Cell 3 has also moved into the field. After 3 h, all three cells can be seen to have moved measurably closer to the needle. The cells move toward the needle at a velocity of  $0.32 \mu\text{m}/\text{min}$ , which is comparable with the velocities reported previously (10).

**In Vivo Cell Collection.** To determine whether cells can be collected from tumors *in vivo* and, if so, if there is a difference in collection efficiency of cells from nonmetastatic and metastatic tumors, experiments were performed by placing needles into the primary tumors generated by either the nonmetastatic MTC-GFP or the metastatic MTLn3-GFP cell lines. For this, a 33-gauge needle (i.d., 102  $\mu\text{m}$ ) was filled as above and inserted into the guide syringe after a blocking wire was removed (as modeled in Fig. 3). The needles were filled with matrigel plus either buffer, 25 nM EGF, or 10% FBS. The 10% FBS was used because the motility of both MTLn3 and MTC cells is stimulated in response to 10% serum (data not shown). After 6 h of collection, needles were withdrawn and the contents of each was extruded into a 35-mm dish containing growth medium, and collected tumor cells were determined by GFP fluorescence. To confirm that only GFP-labeled cells were in the needle, 1  $\mu\text{g}/\text{ml}$  DAPI (4',6-diamidino-2-phenylindole) was added to the dish to stain all cells. All DAPI-stained nuclei were in GFP-labeled cells, indicating that only tumor cells were collected.

The number of cells collected for each condition was normalized to the number of cells collected from the MTC-GFP tumors using needles containing matrigel plus buffer (MEM-BSA) only (Fig. 4).

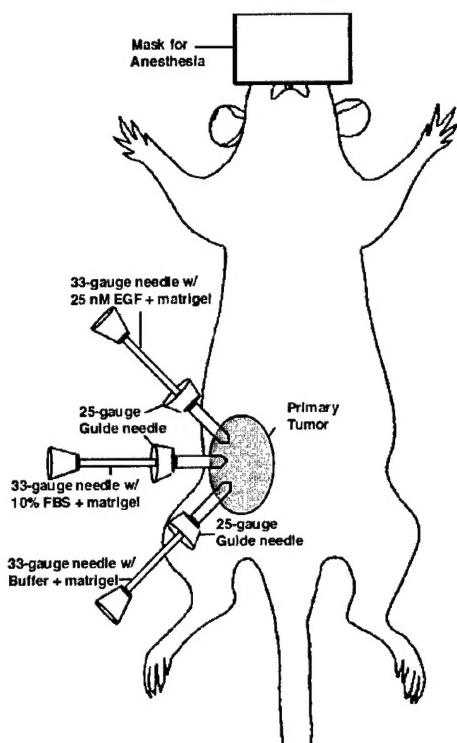


Fig. 3. Method for using needles for *in vivo* cell collection. Needles (i.d., 102  $\mu\text{m}$ ) filled with matrigel and buffer, 25 nm EGF, or 10% FBS are shown placed in 25-gauge guide needles that are inserted into the primary tumor of an anesthetized rat.

For the needle with 25 nm EGF, 15.3 times more MTLn3 cells were collected from metastatic MTLn3 tumors compared with MTC cells from nonmetastatic MTC tumors under the same conditions of collection. In this case, a maximum of 100 cells was collected. Needles containing 10% FBS showed only a 6.0-fold difference between the two tumor types under the same conditions (Fig. 4). There was a 2-fold increase in the number of MTC cells entering the 10% FBS needle from the MTC tumors compared with the number of cells that entered the needle containing only buffer. This difference was shown to be significant (*t* test value, 0.027) and is consistent with the increase in motility of MTC cells when stimulated with 10% FBS *in vitro* (data not shown). We did not attempt to establish long-term cultures of tumor cells collected from the tumors *in vivo* under any of these conditions.

In addition, in needles containing only buffer, 4.3 times more tumor cells were collected from MTLn3 tumors than from MTC tumors (Fig. 4). To determine whether this was due to a cell response to matrigel, a needle was filled with either 1% agarose containing MEM-BSA or 1% agarose containing 10% FBS in MEM-BSA. MTLn3 cells are able to adhere and grow on agarose. However, agarose was chosen because it is a polysaccharide that cannot be degraded by proteases. The number of cells entering the agarose needles was at background for both the 10% FBS-containing needle (data not shown) and the needle with buffer alone (Fig. 4), indicating that either components within the matrigel or the degradation of matrigel provides a chemotactic signal to the cells.

The collection of cells from the MTLn3 tumors was maximal with needles containing 25 nm EGF, resulting in the collection of about 100 cells in 6 h. Because the diameter of the 33-gauge collecting needle is 100  $\mu\text{m}$  and the average cell diameter is 25  $\mu\text{m}$ , the calculated average velocity of cell motility required to account for the collection of 100 cells in 6 h is 0.3  $\mu\text{m}/\text{min}$ . This value is very close to the velocity of cell locomotion observed *in vitro* during chemotaxis (Fig. 2).

## Discussion

In this study, we report that needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor *in vivo* as a pure population suitable for further analysis. Our results demonstrate that tumor cells are collected into needles that have been inserted into a primary tumor when they contain either serum, EGF, or matrigel but not agarose, indicating that a tactic signal is required for collection. The most dramatic accumulation of cells in the needles occurs in response to either EGF or serum. EGF is known to be a chemoattractant for MTLn3 cells (10), whereas serum stimulates the motility of both MTLn3 and MTC cells. However, matrigel was sufficient to collect cells above background, indicating that either the matrigel contains cytokines that are chemotactic for these cells or that limited proteolysis resulting from the interaction of the matrigel with the tumor is sufficient to generate a gradient of chemotactic peptides. Either possibility is consistent with the known properties of matrigel (13–15). Furthermore, MTLn3 cells have a 4-fold greater activity compared with MTC cells (16), which may explain the increase in the number of MTLn3 cells collected into the needles containing matrigel compared with that for MTC cells.

Both EGF and transforming growth factor  $\alpha$  are growth factors found in mammary tissue. MTLn3 cells have around 50,000 EGF receptors/cell, whereas EGF receptors on the MTC cells are not detectable (9). By using EGF as the chemoattractant, we were able to selectively collect 15 times as many metastatic MTLn3 cells from MTLn3-derived metastatic primary tumors as MTC cells from MTC-derived nonmetastatic tumors. Serum, which contains many growth factors with potential chemotactic activity, also stimulated the collection of tumor cells from MTLn3 tumors. Although MTC cells show a motility response to serum, they were not collected with high efficiency into needles in response to serum, indicating that additional factors besides motility are required for the large increase in the number of MTLn3 cells collected in response to serum.

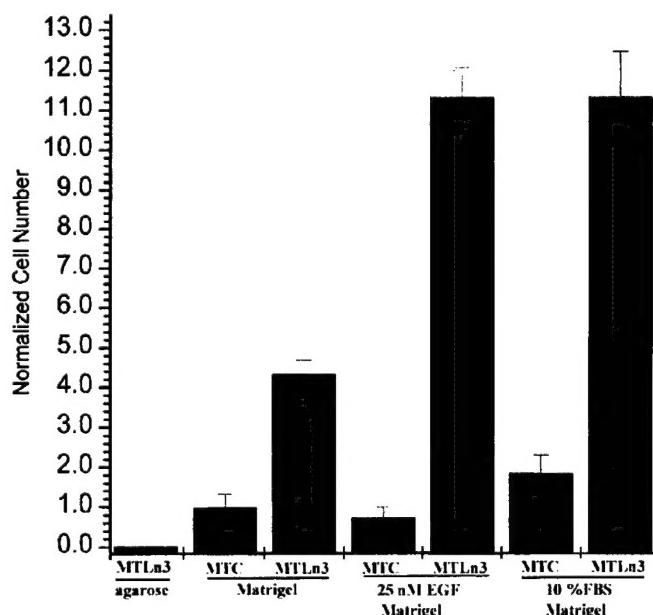


Fig. 4. Metastatic cells (MTLn3) are more efficient than nonmetastatic cells (MTC) at entering matrigel-filled needles in response to EGF *in vivo*. Cells were collected from metastatic (MTLn3) and nonmetastatic (MTC) tumors using the *in vivo* experiment shown in Fig. 3. The maximum response was for cells from the metastatic MTLn3 tumors into EGF- and serum-containing needles. Cells were collected above background from metastatic tumors in response to matrigel in buffer but not agarose. All counts were normalized to MTC cells collected with matrigel in buffer. Bars, the SE of four experiments.

Morphologically, MTC cells are elongated and polarized both *in vivo* and *in vitro*, whereas the MTLn3 cells are generally unpolarized both in culture and in the primary tumor (8, 11). This difference is most dramatically illustrated by using intravital imaging techniques where GFP-expressing tumor cells are imaged directly in the primary tumor (8, 9). *In vivo*, MTLn3 cells are highly polarized around and oriented toward the blood vessels running through the primary tumor. MTC cells, on the other hand, are polarized throughout the tumor, but the polarity is randomly oriented relative to vessels (8, 11).

Characterization of the cells *in vitro* confirms the differences between the two cell lines. In cultures that have not been stimulated with a chemoattractant, MTC cells locomote in a linear direction at approximately twice the velocity of MTLn3 cells. MTLn3 cells, under these conditions, are unpolarized and move in random directions or not at all (11). On stimulation with an EGF gradient, the MTLn3 cells become polarized and move linearly at approximately the same speed as the MTC cells, yet have the ability to reorient themselves to follow an EGF gradient with precision (10), a property not seen in MTC cells.

*In vivo*, in the primary tumor, both cell types move linearly at approximately the same speeds, but the MTLn3 cells tend to move only when they are polarized and in association with a vessel, whereas MTC cells can be seen moving throughout the tumor (8, 9). The ability of the MTLn3 cells to invade into a needle filled with matrigel in response to growth factors is fully consistent with the chemotactic motility exhibited by these cells *in vitro*, their polarity and locomotion toward vessels *in vivo*, and with the dramatically increased efficiency of intravasation measured as blood burden of tumor cells *in vivo* (8). This suggests that chemotaxis may be the key aspect of cell motility that contributes to invasion and intravasation. It also suggests that needles filled with growth factors and matrigel, when inserted into the primary tumor, can faithfully mimic the environment that supports invasion and intravasation *in vivo*, and that the same cell behaviors that contribute to chemotaxis *in vitro* also contribute to invasion *in vivo*.

An advantage of using the needle collection technique described here for the collection of cells for genomic/proteomic analysis is that the cell behavior can be characterized during the collection process. This can be done by varying the conditions required for cell collection such as the extracellular matrix composition and/or cytokines used as chemoattractants, determining how these changes affect efficiency of cell collection, and then relating these observations to the gene expression and protein composition patterns subsequently obtained from array analysis of the collected cells. Furthermore, cells can also be characterized by intravital imaging during collection to directly visualize the cell-cell and cell-extracellular matrix interactions that con-

tribute to the invasion of the needle under these different conditions. In addition, cells could be cultured and transplanted into other host animals to determine whether they stably retain differential characteristics that contribute to metastatic potential. Finally, by comparing the gene expression patterns of cells collected by invasion into needles with that of cells obtained from the whole primary tumor, the blood, and whole metastatic tumors, genes that contribute to the invasive process uniquely may be identified.

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